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The Peptide Antibiotics of *Bacillus*: Chemistry, Biogenesis, and Possible Functions

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INTRODUCTION

In a recent review, Berdy stated that the number of antibiotics produced by members of the genus *Bacillus* was 167 (8). Only a few of the total number elaborated are listed in Table 1. As is generally recognized, these antibiotics are mainly polypeptides. Of this total, 66 different peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis*. Polymyxin and the closely related colistin, bacitracin, the tyrothricin complex (linear gramicidin plus tyrocidine), and gramicidin S have been used, to some extent, for antibacterial therapy. Most of the peptide antibiotics produced by bacilli are active against gram-positive bacteria; however, compounds such as polymyxin, colistin, and circulin exhibit activity almost exclusively upon gram-negative forms, whereas bacillomycin, mycobacillin, and fungistatin are effective agents against molds and yeasts. Although bacilli mainly synthesize peptides, one should not lose sight of the fact that antibiotics belonging to other chemical classes are also produced by these microorganisms (e.g., butirosin [2], an aminoglycoside derived from *Bacillus circulans* and proticin [111], a phosphorus-containing triene elaborated by *Bacillus licheniformis* var. *mesentericus*).

PROPERTIES OF PEPTIDE ANTIBIOTICS

A number of reviews relating to peptide antibiotics has appeared in the literature (14, 15, 55, 71, 72, 80, 109, 117, 132). As a consequence, the properties of peptide antibiotics will be described only briefly below.

(i) Peptide antibiotics are generally much smaller than proteins. Their molecular weights range from 270 (bacilysin) to about 4,500 (licheniformin).

(ii) Generally, a family of closely related peptides rather than a single substance is produced by an organism. The members may differ from each other by one or, at most, a few amino acid residues. In the case of *B. brevis*, both the linear gramicidins (A, B, and C) and the cyclic tyrocidines (A, B, and C) are formed simultaneously. The linear gramicidins (pentadecapeptides) and the tyrocidines (decapeptides) differ markedly in chemical composition, yet within each family the principal variation resides in the aromatic amino acid substitutions of the molecule (34, 73, 114, 131, 134, 135, 136, 137).

(iii) Most of the peptide antibiotics formed by these organisms are composed entirely of amino acids, whereas others may contain amino acids plus other constituents. For example, edeine A contains the base spermidine in addition

TABLE 1. *Some antibiotics elaborated by species of the genus Bacillus*

Species	Antibiotic
Bacillus brevis	Gramicidin S
	Tyrocidine
	Linear gramicidin
	Brevin
	Edeine
	Eseine
	Bresseine
	Brevistin
Bacillus subtilis	Mycobacillin
	Subtilin
	Bacilysin
	Bacillomycin
	Fungistatin
	Bulbiformin
	Bacillin
	Subsporin
	Bacillocin
	Mycosubtilin
	Fungocin
	Iturin
	Neocidin
	Eumycin
Bacillus pumilis	Micrococcin P
	Pumilin
	Tetain
Bacillus mesentericus	Esperin
Bacillus thiaminolyticus	Octopytin (Thianosine)
	Baciphelacin
Bacillus licheniformis	Bacitracin
	Licheniformin
	Proticin
Bacillus polymyxa	Polymyxin
	Colistin
	Gatavalin
	Jolipeptin
Bacillus circulans	Butirosin
	Circulin
	Polypeptin
	EM-49
	Xylostatin
Bacillus laterosporus	Laterosporamine
	Laterosporin
Bacillus cereus	Biocerin
	Cerexin
	Thiocillin

to five amino acid constituents (48). Polymyxins possess 6-methyloctanoic acid or 6-methylheptanoic acid as a fatty acid residue along with a number of amino acid constituents (71, 163).

(iv) Frequently, peptide antibiotics contain amino acids which are unique and are not found in proteins (14, 15, 71). D-Amino acids, basic amino acids (ornithine, diaminobutyric acid), β-amino acids, dehydroamino acids (dehydroalanine), and sulfur-containing amino acids (lanthionine) are often present. In contrast to peptides synthesized by fungi and actinomycetes, peptides from bacilli do not contain *N*-methylamino acid residues.

(v) Most of the peptides are cyclic structures; however, a few are linear (e.g., edeine, linear gramicidins). Besides the cyclic nature of a molecule, there may be unusual linkages or arrangements of the amino acids in the antibiotic. Bacitracin provides an example, for it possesses a thiazoline ring (derived from the condensation of cysteine and isoleucine), a cyclic hexapeptide, as well as an amide bond between the β-carboxyl group of aspartic acid and the epsilon amino group of lysine (1, 167).

(vi) Peptide antibiotics are generally resistant to hydrolysis by peptidases and proteases of animal and plant origin (14, 15, 71, 72, 117), although a few are susceptible to enzymatic attack (polymyxin B by ficin and papain [116]; edeines A and B by carboxypeptidase [49]; bacilysin by leucine aminopeptidase and Pronase [124]; gramicidin S by subtilopeptidase A [174]).

FORMATION OF ANTIBIOTICS IN RELATION TO GROWTH

A number of investigations have shown that the synthesis of peptide antibiotics is initiated after the organism has passed the rapid growth phase. This was noted with gramicidin S (160), tyrocidine (36, 90), polymyxin (116), edeine (82), bacitracin (11), mycobacillin (5), and bacilysin (124, 126). Tomino et al. (160) demonstrated that gramicidin S and the enzymes necessary for its synthesis were produced by *B. brevis* during the late logarithmic phase of growth (107, 160). The enzymatic activity for antibiotic synthesis then disappeared within a few hours. Similar results were observed with tyrocidine synthetase (36). The reason for the loss of enzyme activity has not been established conclusively. Recently, Lee et al. (90) observed that the tyrocidine-forming enzymes disappeared from the soluble portion of the cell and became associated with the forespore membrane during stage IV of sporulation. However, even this membrane-bound activity disappeared soon thereafter. Demain et al. (25) found

that the disappearance of gramicidin S synthetases in *B. brevis* is due to an oxygen-dependent inactivation.

Although antibiotic formation usually follows logarithmic growth (presumably due to some type of repression of antibiotic synthetases in the growth phase), this is not universally observed. It is clear that antibiotics are sometimes produced during growth and that both genetic and nutritional modifications can shift the time of antibiotic synthesis in relation to the growth phase (23). It is quite possible that peptide antibiotic formation is controlled by carbon and nitrogen catabolite repression or is under growth rate control (106); manipulations which affect these controls would be expected to modify the temporal relationship between antibiotic synthesis and growth. In this regard, production of bacitracin by *B. licheniformis* parallels growth in a chemically defined medium (47, 152). However, Haavik (41, 42) reported that when glucose was added to a defined medium, bacitracin synthesis was inhibited during the first few hours of growth. He initially concluded that this delay in antibiotic production was not due to carbon catabolite repression but to the low pH created by the organism's metabolism. His conclusion was based on the reversal of the glucose effect by added $CaCO_3$ (41). However, he later (42) found that the depression of bacitracin synthesis was not due to pH per se but to the acetic and pyruvic acids produced from the glucose, i.e, these acids were only inhibitory when undissociated and thus neutralization eliminated their inhibition. Lowering pH with HCl or phthalic acid did not inhibit bacitracin formation. It should be emphasized that this work does not eliminate the possibility that repression of bacitracin synthetase is due to the undissociated organic acids produced by glucose catabolism.

In addition to bacitracin, the production of tyrocidine (79), linear gramicidins (79), and polymyxin E (colistin) (28) has also been observed to parallel active growth of the producing bacilli under certain conditions. Moreover, gramicidin S (13) and its synthetases (106) can be synthesized by exponentially growing cells in continuous culture. Thus, it is clear that peptide antibiotics can be synthesized during active growth. Whether they function in the producing cells' metabolism at some stage during growth, as some investigators (43, 52, 70) have considered, will be discussed in a later section of this review.

CELL-FREE SYNTHESIS OF PEPTIDE ANTIBIOTICS

The cell-free synthesis of gramicidin S, tyrocidine, linear gramicidin, edeine, bacitracin, colistin, and mycobacillin has been reviewed (15, 55, 72, 80, 117). These biosynthetic systems are quite distinct from those synthesizing proteins. The requirements for antibiotic synthesis are fairly similar and generally include the requisite amino acids of the peptide antibiotic in question, adenosine 5'-triphosphate (ATP), Mg^{2+} ion, a reducing agent, and the particle-free supernatant fluid. Many of the synthetases have been extensively purified by conventional procedures, and considerable success has been achieved in resolving the polyenzyme complexes by column chromatography (diethylaminoethyl [DEAE]-cellulose, hydroxylapatite, Sephadex G-200). The peptide-synthesizing systems have proven to be insensitive to chloramphenicol, puromycin, deoxyribonuclease, and ribonuclease (RNase), and it is quite clear that transfer ribonucleic acid (tRNA), messenger RNA (mRNA), and ribosomes are not directly involved in the biosynthetic process. The contributions of several laboratories have led to our present understanding of the mechanism of biosynthesis of gramicidin S and tyrocidine (9, 12, 35-38, 65, 75, 81, 87, 88, 94, 95, 97, 113, 119, 133, 160, 162, 175). Far less is known, however, regarding the cell-free synthesis of other peptide antibiotics produced by bacilli.

ENZYMATIC SYNTHESIS OF GRAMICIDIN S

Gramicidin S (Fig. 1), formed by certain strains of *B. brevis*, is a cyclic decapeptide consisting of two identical pentapeptides, D-phenylalanyl-L-prolyl-L-valyl-L-ornithinyl-L-leucine (15, 55, 71). The presence of D-phenylalanine and L-ornithine, constituents which are not found in proteins, is noteworthy.

In the formation of gramicidin S in vivo, the

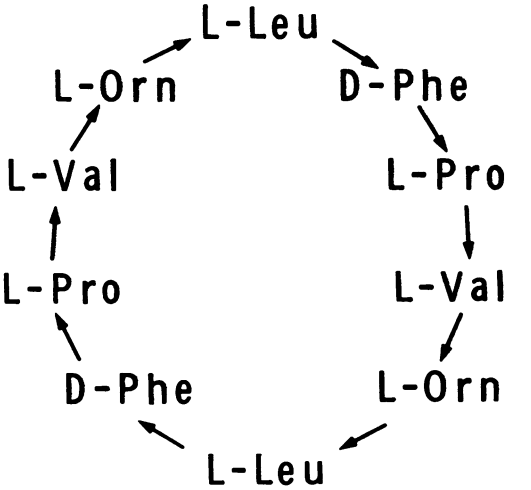

FIG. 1. *Gramicidin S.*

first amino acid added is phenylalanine; in vitro it can be replaced by tyrosine, but not by tryptophan (128). Phenylalanine (or the tyrosine replacing it) is incorporated as the D-amino acid. An enzyme (designated as fraction II or the light enzyme) with a molecular weight of 100,000 (Table 2) activates L-phenylalanine (or D-phenylalanine) and racemizes it (37, 38, 160, 171, 172). Activation (Fig. 2) is an ATP-dependent process and, as observed in protein synthesis, there is formation of a phenylalanyl adenylate-enzyme complex (37, 75, 160, 171, 172). The remaining four amino acids, L-proline, L-valine, L-ornithine, and L-leucine, are activated by a second protein designated fraction I or heavy enzyme (Table 2) which has a molecular weight of 280,000 (37, 75, 160). In addition to the amino acids, ATP and Mg^{2+} ion are requirements for the activation step. Each of the four amino acids is activated independently on separate sites of the polyenzyme, also being bound as aminoacyl adenylates (Fig. 2). The activation process, as measured by the ATP-inorganic pyrophosphate (PP_i) exchange reaction, appears to be relatively specific since only amino acids chemically related to those in gramicidin S are activated. In contrast to the ribosomal system, acylation of tRNA does not take place during activation with either the light or the heavy enzyme (80, 87, 94, 97).

Both enzymes have been shown to catalyze not only an ATP-PP_i exchange reaction, but an ATP-adenosine 5'-monophosphate (AMP) exchange as well with the appropriate amino acid substrates. This is due to the presence of secondary acceptors of the bound amino acids on the enzymes (37, 38). The aminoacyl moiety is transferred from the adenylate site to an SH group on the enzyme, yielding a covalently bound thioester-linked amino acid. Thus, the heavy enzyme contains four activating enzymes organized into a tightly associated multienzyme complex; the binding of the amino acid substrates to this complex exhibits a 1:1:1:1 stoichiometry (75). Both the adenylate and SH sites will be occupied under saturating conditions, because once the amino acid is transferred to a thiol group, the adenylate site is free to accept another molecule of activated amino acid (75, 94, 97).

Several experiments have provided support for the view that covalent thioester bonds are formed between the individual amino acids and the specific enzymes involved (32, 38, 88, 129).

TABLE 2. *Features of gramicidin S and tyrocidine synthetases*

Enzyme	Fraction	Nomenclature	Amino acid activated	4'-Phosphopantetheine	Mol wt
Gramicidin S synthetase	II	Light enzyme	L- and D-phenylalanine	0	100,000
	I	Heavy enzyme	L-Proline, L-valine, L-ornithine, L-leucine	1	280,000
Tyrocidine synthetase	I	Light enzyme	L- and D-phenylalanine	0	100,000
	II	Intermediate enzyme	L-Proline, L- and D-phenylalanine (tryptophan)	1	230,000
	III	Heavy enzyme	L-Asparagine, L-glutamine, L-phenylalanine (tryptophan, tyrosine) L-valine, L-ornithine, L-leucine	1	460,000

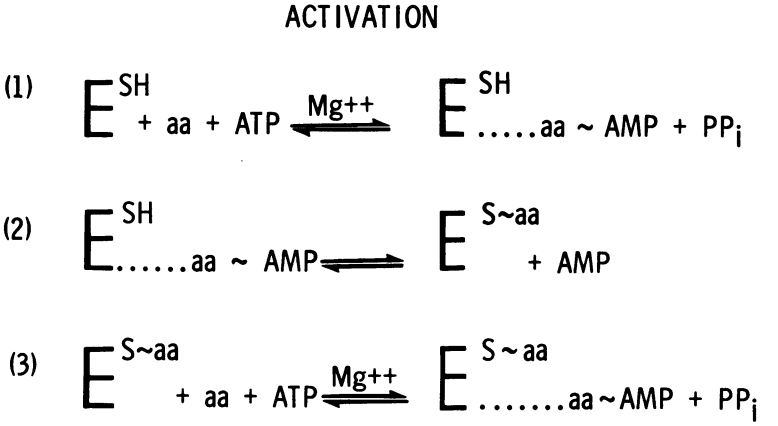

FIG. 2. *Proposed mechanism of activation of amino acids in gramicidin S. (Reprinted with permission of F. Lipmann.)*

(i) After an incubation, an activated amino acid-enzyme complex can be recovered by Sephadex G-50 chromatography; precipitation of such a complex with trichloroacetic acid releases all of the bound adenylate, but only one-half of the bound amino acid is liberated. (ii) Dilute alkali (at pH 9 to 10) or treatment with mercuric salts at neutral pH brings about a quantitative discharge of the remaining amino acid from the trichloroacetic acid precipitate. (iii) Hydroxylamine treatment of the precipitate will also release the amino acid as the hydroxamate. (iv) Treatment of the precipitate with sodium borohydride reductively cleaves the bond with release of the amino acid as the corresponding amino alcohol. (v) Oxidative cleavage with performic acid also will liberate the amino acid. Thus, the behavior shown by the covalently bound amino acid to acid, base, mercuric salts, etc., comprises all of the characteristic properties of a thioester linkage.

Initiation of peptide bond synthesis. It is now well established that free peptide intermediates are not participants and that an enzyme-bound, activated D-phenylalanine residue is the starting point for gramicidin S formation (32, 38, 53, 98). The direction of peptide chain growth is from the N-terminal to the C-terminal end as in protein synthesis, and only enzyme-bound intermediates are involved. These conclusions were based on in vivo (53, 159) and in vitro (16, 38, 75) experiments. During gramicidin S synthesis, the amino acids are added singly and in a fixed order until a pentapeptide is formed; omission of one amino acid interrupts the elongation process.

For peptide synthesis to occur, the charged or activated enzymes have to be combined (75). The first peptide bond involves thioester-bound D-phenylalanine on the light enzyme and thioester-linked proline on the heavy enzyme (74, 81, 87, 94, 95, 97). The carboxyl-activated D-phenylalanine is transferred to the imino group of proline on the heavy enzyme (Fig. 3). In turn, the thioester-linked dipeptide is transferred to the next thioester-linked amino acid, L-valine, to form a tripeptide. All peptides remain attached to the enzyme until leucine, the last amino acid in the sequence, is added (Fig. 4). Leucine addition brings about a rapid release of cyclic gramicidin S from the polyenzyme. The mechanism of the cyclization step is not well understood. Lipmann's group proposed that the cyclic decapeptide of gramicidin S may arise by an antiparallel doubling reaction between two carboxyl-activated pentapeptide units presumably attached to different heavy enzyme components (94, 97) (Fig. 4). Some evidence, however, suggests an intramolecular cyclization (87, 171). Stoll et al. (153) reported that, after the first pentapeptide chain is formed, it is transferred to a holding site (—SH group) on the heavy enzyme. When the second pentapeptidyl unit is synthesized subsequently, the two pentapeptide chains attached to the same heavy enzyme cyclize by head-to-tail condensation to yield a molecule of gramicidin S (Fig. 5).

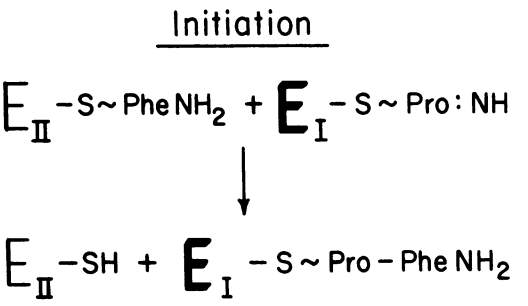

FIG. 3. *Initiation reaction for gramicidin S formation. (Reprinted with permission of F. Lipmann.)*

4'-Phosphopantetheine, bound to the heavy enzyme of gramicidin S synthetase, mediates the biosynthesis of the tri-, tetra-, and pentapeptides in alternative transthiolation and transpeptidation reactions (38, 39, 76, 77, 87, 94, 95, 97, 98). 4'-Phosphopantetheine is conceived to function as a swinging arm in which it alternates as acceptor of the growing peptide chain and donor of the peptide to the next thioester-linked amino acid in sequence (87, 95, 119). These reactions continue until the tetrapeptide is linked to the terminal amino acid, leucine, to complete the nascent pentapeptide chain. Lipmann has postulated that the thioester-linked polymerization of polypeptides may have evolved from thioester-linked fatty acid synthesis (94, 96).

ENZYMATIC FORMATION OF TYROCIDINE

The mechanism of biosynthesis of tyrocidine is similar to that of gramicidin S (80, 94, 95, 97), and only a brief discussion will be presented here. Tyrocidines A, B, and C are cyclic decapeptides (Fig. 6) (34, 71, 73, 114, 131) produced by the same strains of *B. brevis* that elaborate the linear gramicidins. This strain cannot produce the cyclic gramicidin S. Although they are cyclic decapeptides like gramicidin S, tyrocidines possess a somewhat different sequence of amino acids. The biosynthesis of tyrocidine also begins with D-phenylalanine and continues with proline (33, 35, 36, 89, 91, 94, 95, 97, 119, 128). However, five different amino acids, L-phenylalanine, D-phenylalanine, L-asparagine, L-glutamine, and L-tyrosine, follow. After tyro-

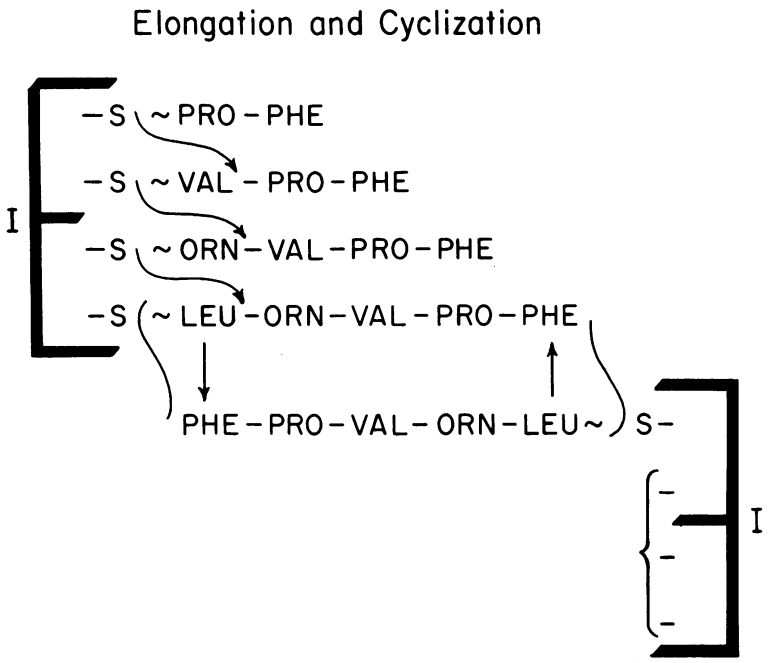

FIG. 4. *Proposed mechanism of elongation and cyclization for gramicidin S biosynthesis. (Reprinted with permission of F. Lipmann.)*

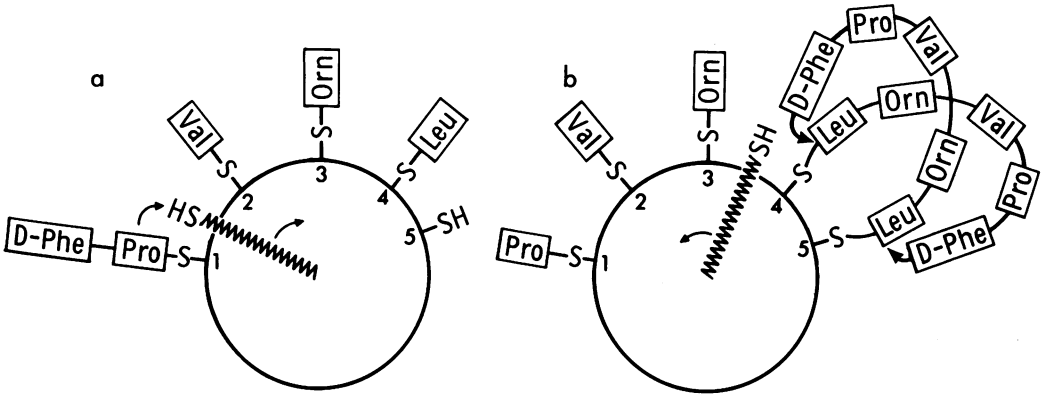

FIG. 5. *Model for the role of enzyme I in gramicidin S biosynthesis. Positions 1, 2, 3, and 4 are occupied by thioester-linked proline, valine, ornithine, and leucine, respectively. Position 5 is envisioned as the thiol "waiting" site for the pentapeptide. The zigzag line represents the pantetheine arm with its terminal SH group. (A) Growth of nascent peptide chain. (B) Head-to-tail cyclization reaction involving two identical pentapeptides. (Reprinted with permission of S. G. Laland.)*

sine, the sequence of gramicidin S is seen again with L-valine, L-ornithine, and then L-leucine being the last amino acid added.

Purification of the tyrocidine-synthesizing system yields three complementary enzymes: a light fraction (molecular weight 100,000), an intermediate one (molecular weight 230,000) and a heavy enzyme (molecular weight 440,000) (22, 36, 69, 91, 127). The amino acids activated by each of these fractions are shown in Table 2. The light enzyme activates and racemizes phenylalanine (tyrosine or tryptophan can replace it) (97, 127). The intermediate enzyme activates proline, phenylalanine, and phenylalanine in positions 2, 3, and 4, respectively. Phenylalanine at site 4 is also racemized on the enzyme. The heavy enzyme catalyzes the activation of the remaining six amino acids. If one divides the molecular weight of the heavy enzyme of the gramicidin S synthetase complex or the intermediate or heavy enzyme of tyrocidine synthetase by the number of amino acids activated, one obtains a value of 70,000 to 75,000 daltons per amino acid activated and thioesteri-

fied (89, 91, 95). The intermediate and heavy enzymes contain 1 mol each of 4'-phosphopantetheine bound per mol of enzyme protein. In tyrocidine synthesis, 4'-phosphopantetheine also functions in the alternative transthiolation and transpeptidation reactions during nascent peptide elongation (76, 77, 89, 128). Figure 7 schematically depicts the mechanism of initiation, elongation, and cyclization of the decapeptide of tyrocidine. Initiation of chain growth is similar to that seen in gramicidin S biosynthesis. Addition of amino acids occurs singly,

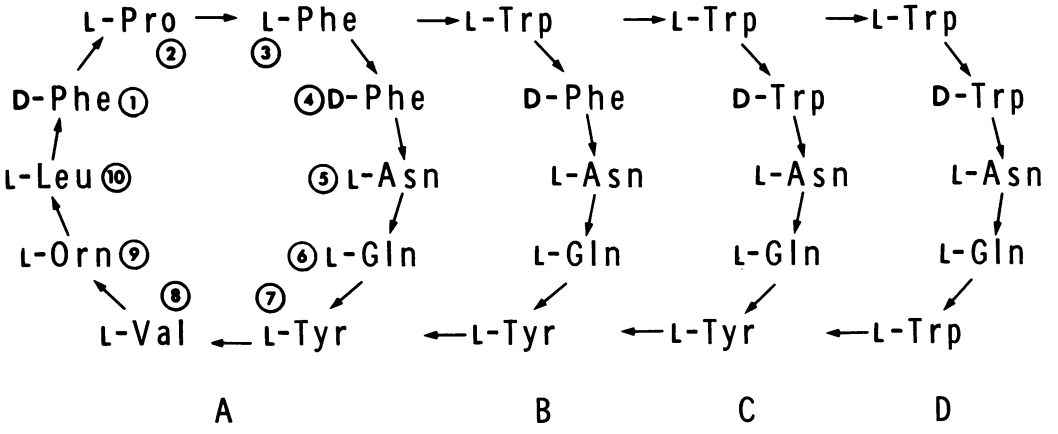

FIG. 6. *Amino acid sequence of tyrocidines A, B, C, and D. The left half sequences, omitted in the diagrams of tyrocidines B, C, and D, are identical to that of tyrocidine A.*

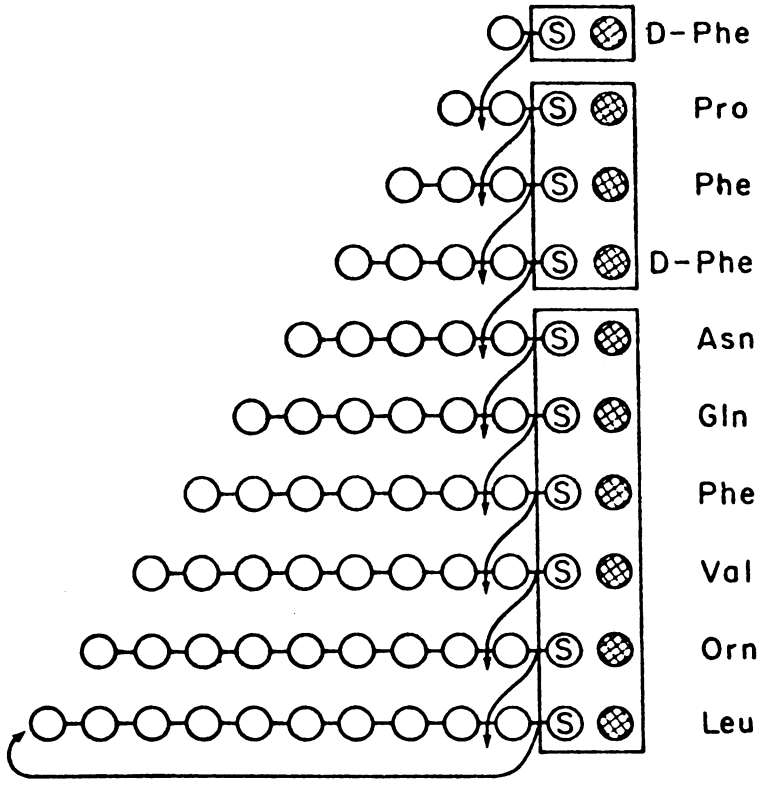

FIG. 7. *Summary of reactions involved in tyrocidine biosynthesis. The cross-hatched areas indicate individual sites for binding of the aminoacyl adenylates, each next to thiol groups responsible for thioester binding of amino acids and peptides as shown. Phosphopantetheine arm is omitted. (Reprinted with permission of F. Lipmann.)*

with a tetrapeptide being formed on the intermediate enzyme. The tetrapeptide is then transferred to the heavy enzyme where the remaining six amino acids are added sequentially to complete the peptide chain (127, 128). Cyclization of the tyrocidine decapeptide appears to be a relatively slow reaction, the linear decapeptide having been isolated from the heavy enzyme and characterized. Cyclization involves the activated enzyme-bound thioester-linked carboxyl group of leucine and the free amino group of D-phenylalanine. As with gramicidin S synthesis in vitro, omission of one amino acid in the sequence halts further chain elongation.

Disaggregation of the intermediate and heavy polyenzymes into subunits (89, 91, 95). Each of the polyenzyme components of the tyrocidine-synthesizing system appears to consist of subunits and contains 1 mol of covalently bound 4'-phosphopantetheine per mol of polyenzyme. On the basis of dissociation experiments, Lee et al. (89, 91) confirmed that the intermediate enzyme comprises three, and the heavy enzyme comprises six, amino acid-activating subunits of the 70,000-dalton size. The subunits retained the ability to catalyze ATP-PP_i exchanges and the thioesterification reactions with the appropriate amino acid substrates, but were unable to carry out the polymerization step. Upon dissociation, both polyenzymes also yielded a pantetheine-containing protein fraction with a molecular weight of 17,000 (77, 89, 91, 95). Evidence that 4'-phosphopantetheine participates in the polymerization reaction was provided also by the dissociation experiments. After incubation of lysates of *B. brevis* or purified polyenzymes with amino acid substrates, the polyenzymes (from the crude or purified enzyme system) were degraded into subunits and subsequently purified. In both cases, a product containing both pantothenic acid and a nascent tyrocidine peptide was recovered (89, 95). These studies demonstrate that the intermediate and heavy enzymes of tyrocidine synthetase consist of specific amino acid-activating subunits (molecular weight 70,000) and a pantetheine-bearing protein of 17,000 daltons which functions in the biosynthesis of the nascent tyrocidine peptide chain (77).

LINEAR GRAMICIDINS

The gramicidins are linear pentadecapeptides produced by the same strain of *B. brevis* that elaborates the tyrocidines (Fig. 8). Gramicidins A, B, and C are a mixture of valine and isoleucine gramicidins (134, 137). They differ only in the aromatic amino acid (tryptophan, tyrosine, or phenylalanine) incorporated at position 11 of the peptide. The amino terminal L-valine (or L-isoleucine) is formylated and the carboxyl terminal tryptophan is linked to the amino group of ethanolamine. It should be noted that the peptide molecule consists almost entirely of alternating L- and D-amino acid residues.

Bauer et al. (7) were able to achieve a partial biosynthesis of linear gramicidin, using a solu-

COMPOSITION OF GRAMICIDIN

Compd.	Positions 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
I	Valine-gramicidin A: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH
II	Isoleucine-gramicidin A: HCO-L-Ileu-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH
III	Valine-gramicidin B: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Phe-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH
IV	Isoleucine-gramicidin B: HCO-L-Ileu-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Phe-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH
V	Valine-gramicidin C: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Tyr-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH
VI	Isoleucine-gramicidin C: HCO-L-Ileu-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Tyr-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH

FIG. 8. *Composition of linear gramicidins A, B, and C.*

ble enzyme system obtained from extracts of *B. brevis* that had been purified through ammonium sulfate fractionation and Sephadex G-200 column chromatography. The location of the linear gramicidin-synthesizing enzymes was followed by their amino acid-dependent ATP-$^{32}PP_i$ exchange activities. The fractions that activated the amino acids incorporated into linear gramicidin were devoid of amino acid-tRNA ligase activity. However, due to its instability, further resolution of the linear gramicidin synthetase into complementary enzyme fractions was not attempted. Despite these difficulties, the enzyme system catalyzed the synthesis of an enzyme-bound pentadecapeptide. Chemical ethanolaminolysis liberated the peptide from the enzyme, and subsequent chemical formylation of the N-terminal valine yielded a compound that appeared to be chromatographically identical to authentic linear gramicidin. Bauer et al. (7) noted that enzymatic formylation of the N-terminal valine could only be achieved with crude extracts derived from *B. brevis*. Unfortunately, attempts to add ethanolamine to the carboxyl terminal tryptophan enzymatically to complete the biosynthesis of linear gramicidin were unsuccessful.

All the amino acid precursors of linear gramicidin can be incorporated into a protein-bound thioester-linked peptide which proved to be stable to acid, but liberated by alkali (pH 11) or peroxidation (7). The stoichiometry of the amino acids incorporated into the peptide as deduced from double-labeling experiments was 1 Gly:2 Ala:4 Leu:4 Val:4 Phe in agreement with the stoichiometry of the amino acids in linear gramicidin. Alanine was found only in the L-configuration, and valine was present equally as D and L, whereas leucine was only found in the D-configuration, which correlates rather well with the optical configuration of the amino acids in linear gramicidin. These results clearly suggest that a pentadecapeptide which remains thioester-linked to the enzyme is biosynthesized. The peptide may be released enzymatically by aminoethanolysis. The stage during biosynthesis at which formylation of N-terminal valine occurs remains uncertain. However, Bauer et al. (7) suggested that formylation (presumably requiring tetrahydrofolate) most likely is carried out after synthesis of the polypeptide is completed. The participation of 4'-phosphopantetheine as a cofactor in linear gramicidin formation is anticipated, but this point has not been established to date.

BACITRACINS

The bacitracins are synthesized by certain strains of *B. licheniformis* (167). Although several bacitracins have been described in the literature, the most completely investigated is bacitracin A (Fig. 9), a dodecapeptide with four of the amino acids (glutamic, ornithine, phenylalanine, and asparagine) in the D-configuration (1, 22, 99, 100). As already noted, the molecule contains a cyclic hexapeptide and a thiazoline ring structure.

Several laboratories have reported the cell-free synthesis of bacitracin (30, 31, 56–58, 118, 148, 149). Purified bacitracin synthetase (molecular weight 800,000) has been resolved into three complementary fractions by means of Sepharose affinity column chromatography (30) or a combination of hydroxyapatite column chromatography and sucrose density gradient centrifugation (56). It appears that all three fractions are required for maximal bacitracin synthesis; a combination of any two compo-

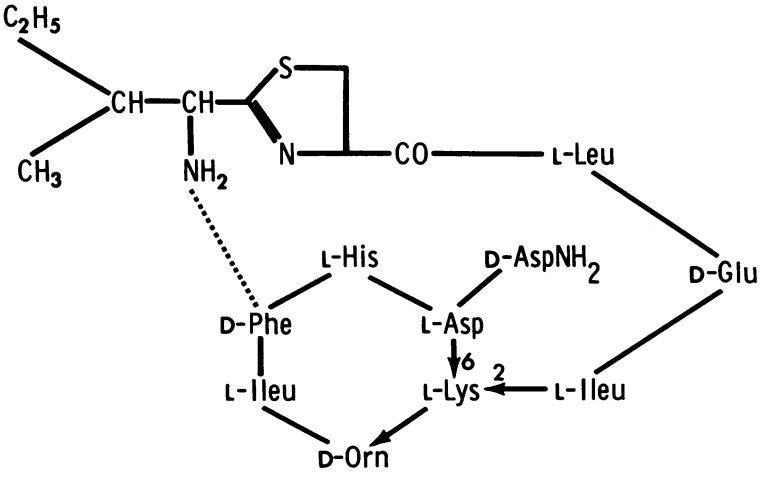

FIG. 9. *Bacitracin A.*

nents is less than 25% as active as the three fractions together. The enzyme in peak A (designated component I) has a molecular weight of 200,000 and activates L-isoleucine, L-cysteine, L-leucine, and L-glutamic acid. A second fraction, B (component II), has a similar molecular weight (210,000) but only activates L-lysine and L-ornithine, whereas the third fraction, C (component III), possesses a molecular weight of 380,000 and activates L-phenylalanine, L-histidine, L-aspartic acid, L-asparagine, and L-isoleucine (or L-valine). Amino acid activation requires ATP and Mg^{2+}, and it has been noted that aminoacyl adenylates are formed; subsequently, the amino acids are thioester-bound to the enzyme before polymerization occurs on the polyenzyme. Froyshov and Laland (31) first presented evidence that pantothenic acid is present in bacitracin synthetase and concluded that 4'-phosphopantetheine is bound to the synthetase. Recently, Ishihara et al. (56) extended this observation and reported that each of the three components may contain one equivalent of covalently bound 4'-phosphopantetheine. These components are relatively impure (40 to 70%), so that the results should be considered as somewhat preliminary in nature.

Earlier studies with intact cells, protoplast preparations, and in vitro systems indicated that D-amino acids could not replace their respective L-enantiomorphs as precursors for bacitracin formation (11, 21, 56, 57, 118, 151). However, studies with the partially purified enzyme preparation revealed that the L-enantiomorph of glutamic acid, phenylalanine, and asparagine can be replaced, to a considerable extent, by the D-isomers (glutamic acid, 27%; phenylalanine, 79%; asparagine, 81%) for antibiotic synthesis (31). By contrast, D-ornithine could not substitute for its L-isomer. It will be of considerable interest to learn the detailed mechanism of bacitracin biosynthesis as there are obvious differences as well as similarities between the structure of this molecule and those of gramicidin S and tyrocidine.

EDEINE

The edeines (Fig. 10) are strongly basic, linear oligopeptide antibiotics produced by *B. brevis* strain Vm4 (48, 50, 82, 125). The amino acid composition is unusual; besides glycine, the molecule consists of isoserine, β-tyrosine, α,β-diaminopropionic acid, and 2,6-diamino-7-hydroxyazaleic acid. The glycine residue in edeine A is attached to the basic constituent, spermidine. In the case of edeine B, the base is guanylspermidine (*N*-guanyl-*N'*-(3-aminopropyl)-1,4-diaminobutane). The structure of edeine D is similar to edeine A with β-phenyl-β-alanine substituting for β-tyrosine (169).

Although a number of papers have appeared on the cell-free synthesis of edeine (82, 84–86), the mechanism of its biosynthesis is not fully understood. Polyenzyme fractions that are specific for the activation and polymerization of the constituent amino acids of edeine have been isolated from *B. brevis* Vm4 by Kurylo-Borowska (85). The size and number of fractions that are resolved by DEAE-cellulose column chromatography and Sephadex G-200 sieving appear to vary with the age of the culture and method of extract preparation (e.g., lysozyme treatment, grinding). However, only two polyenzyme fractions (designated A and B) are needed for edeine formation in the presence of constituent amino acids, spermidine (or guanylspermidine), ATP, and Mg^{2+}. Fraction C, which may be a disaggregation product of the more complex polyenzyme B fraction, enhances the formation of edeines A and B. The molecular weight of these fractions is reported to be 210,000 (fraction A), 180,000 (fraction B), and 100,000 (fraction C) (79). Fraction A activates only β-tyrosine; fraction B catalyzes the activation of the other four constituent amino acids of edeine (isoserine, 2,6-diamino-7-hydroxyazaleic acid, α,β-diaminopropionic acid, and glycine). Fraction C activates isoserine and diaminopropionic acid as well as certain nonconstituent amino acids such as L-α- and L-β-alanine and L-leucine. Fractions A and B also catalyze ATP-AMP exchanges, suggestive of secondary acceptor sites for the amino acids on the polyenzyme complexes. Fractions A and B were shown to contain covalently bound pantetheine. There is no information concerning the sequence of addition of amino acids during nascent peptide synthesis, nor are there any data regarding the mechanism of attachment of the base to glycine during edeine formation. The large size of frac-

EDEINE

β-TYR—β-SER—DAPA—DAHAA—GLY—(GUANYL) SPERMIDINE

FIG. 10. *Structure of edeines A and B. Abbreviations: β-TYR, β-tyrosine; β-SER, β-serine (isoserine); DAPA, diaminopropionic acid; DAHAA, diaminohydroxyazelaic acid; GLY, glycine. In edeine B, guanylspermidine substitutes for spermidine present in edeine A.*

tion A is surprising in light of the fact that it only activates β-tyrosine. A separate enzyme, tyrosine α,β-amino mutase (molecular weight 75,000) catalyzes the conversion of L-α-tyrosine to L-β-tyrosine (84).

MYCOBACILLIN

Mycobacillin (Fig. 11) is a cyclic peptide antibiotic that contains 13 residues of 7 different amino acids (6, 103, 144). The sequence and linkages of the amino acids in the molecule were recently elucidated (103, 144). Mycobacillin lacks free amino groups but contains two free α-carboxyl groups. All of the aspartic acid residues of the antibiotic have been shown to be in alpha-peptide linkage; the glutamic acid residues (D-isomers), however, are gamma-linked to the adjacent amino acid. Sengupta et al. (144) demonstrated that both L-aspartic acid (position 5) and D-aspartic acid (positions 2, 8, 11, and 13) are present.

A 10,000 × *g* supernatant fraction obtained from the protoplast lysate of *B. subtilis* B_3 was reported to catalyze the synthesis of the antibiotic (145). The process required ATP and was insensitive to the action of RNase and inhibitors of protein synthesis such as streptomycin, chloramphenicol, and puromycin.

There appear to be certain features which distinguish mycobacillin formation from the biosynthesis of other peptide antibiotics (gramicidin S, tyrocidine, bacitracin, edeine). For example, the activation step may involve an ATP-inorganic orthophosphate (P_i) exchange reaction. Sengupta and Bose noted that such an exchange occurred with L-proline, a constituent amino acid of mycobacillin (146). This exchange was further stimulated by the other amino acid components of the antibiotic. Omission of certain of the constituent amino acids (D-aspartic acid, D-glutamic acid, L-tyrosine, and L-serine) diminished the stimulation observed. Pyrophosphate, an inhibitor of gramicidin S formation, stimulated mycobacillin synthesis in vitro. It was also reported that several peptides were synthesized as intermediates in the biosynthesis of the antibiotic in vitro (146). Peptides

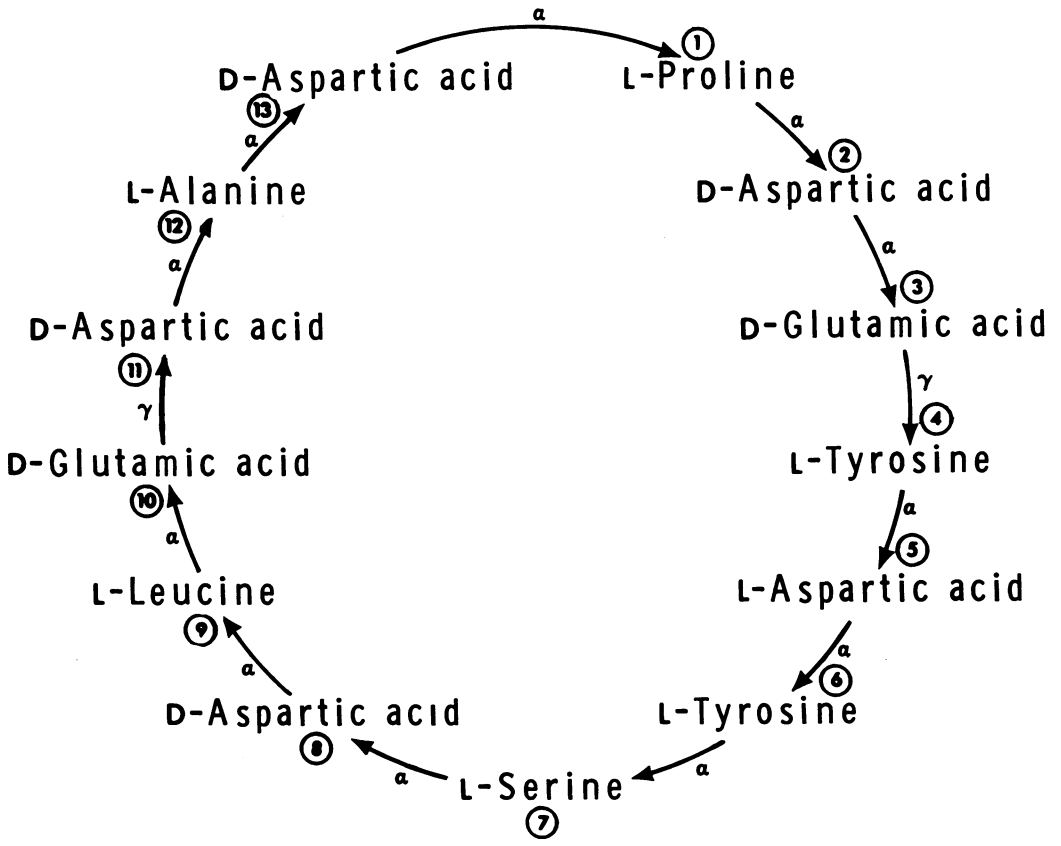

FIG. 11. *Structure of mycobacillin.*

containing two, three, four, five, and six different amino acids were formed after incubation with proline plus one to six additional amino acid components of the mycobacillin molecule. No peptide containing all seven different mycobacillin amino acids was observed, nor was any straight-chain tridecapeptide obtained, suggesting that cyclization may occur immediately after such a peptide is synthesized. Characterization of the peptides revealed that each compound had proline as its N-terminal residue and, depending on the peptide in question, aspartic acid, glutamic acid, or tyrosine as its C-terminal amino acid. The peptides were di-, tri-, hexa-, octa-, and undecapeptides; however, the total sequence as well as the stereochemistry of the constituent amino acids in these peptides was not established. The participation of such compounds as intermediates in the biosynthesis of mycobacillin is certainly suggested by the data presented, yet very little is known concerning the detailed mechanism of synthesis of this antifungal antibiotic, particularly with regard to the substrate and cofactor requirements, the nature of the activation, initiation, elongation reactions, and the cyclization of the nascent linear mycobacillin peptide. It would be of interest to know whether, during antibiotic synthesis, the peptide intermediates are enzyme-bound or free. Also, the nature and properties of the polyenzyme systems that catalyze the polymerization reactions are not well known. The answer to these as well as other questions pertaining to the biosynthesis of mycobacillin must await additional purification of the enzyme system(s) involved.

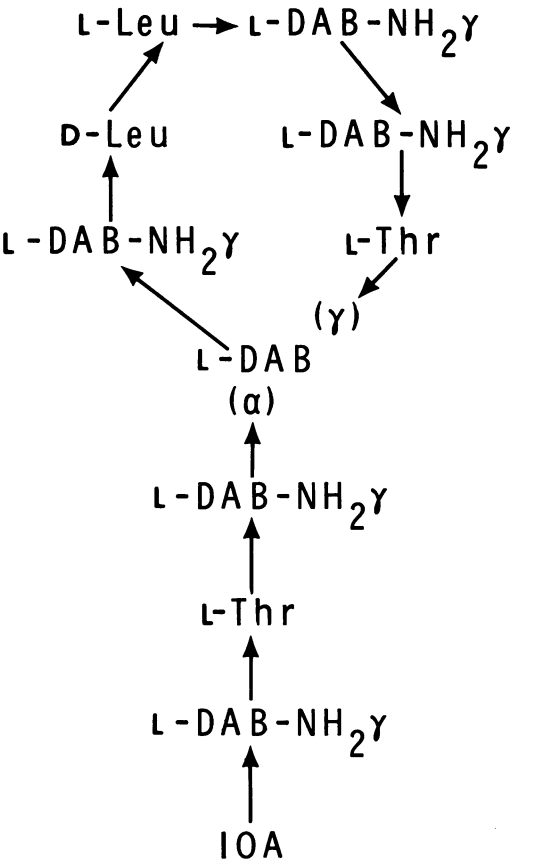

FIG. 12. *Structure of colistin B. Abbreviations: IOA, isooctanoic acid; DAB, diaminobutyric acid; Thr, threonine; Leu, leucine. In colistin A, the fatty acid present is 6-methyloctanoic acid.*

POLYMYXIN AND COLISTIN

Polymyxin is produced by strains of *Bacillus polymyxa* and the related colistins are elaborated by *Bacillus colistinus* Koyama (115, 155–157, 163). Structurally similar to the polymyxins and colistins are the circulins (115). These antibiotics are branched, cyclic decapeptides linked to a fatty acid residue. For example, 6-methyloctanoic acid or isooctanoic acid, present in colistins A and B, respectively, is present in α-amide linkage with the terminal α,γ-diaminobutyric acid residue (155, 157). The structure of colistin B is presented in Fig. 12. It is of interest to point out that 6-methyloctanoic acid and isooctanoic acid are synthesized from isoleucine and valine, respectively (59). Two compounds, N^{α}-6-methyloctanoyl-α,γ-diaminobutyric acid and N^{α}-isooctanoyl-α,γ-diaminobutyric acid, formed by colistin-producing cells, have been implicated as intermediates in the biosynthesis of colistins A and B, respectively (60). The cell-free synthesis of colistins A and B has been achieved using soluble extracts obtained from *B. colistinus* and an incubation mixture consisting of N^{α}-6-methyloctanoyl-α,γ-diaminobutyric acid (and N^{α}-isooctanoyl-α,γ-diaminobutyric acid), leucine, α,γ-diaminobutyric acid, [^{14}C]threonine, ATP, and Mg^{2+} (61, 62). The radioactive product formed was identified as colistin by paper chromatography. These studies suggest that the fatty acyldiaminobutyrate intermediate may initiate colistin biosynthesis. As observed earlier in the case of polymyxin formation in vivo (115), synthesis of colistin in vitro was insensitive to chloramphenicol and puromycin (62). Enzyme activity for colistin synthesis was observed chiefly in the soluble fraction (100,000 × *g* supernatant fluid) of the cell. Additional evidence that the polymerization process is distinct from protein synthesis was the finding that antibiotic synthesis was not inhibited by RNase (62).

An activating enzyme which may function in polymyxin biosynthesis was partially purified from sonic extracts of the polymyxin B-producing organism, *B. polymyxa* 2459 (66). The enzyme catalyzes an L-α,γ-diaminobutyrate-dependent exchange of ATP with PP_i. The specific activity of this enzyme increased approximately 10-fold during vegetative growth, which correlated with the appearance of the antibiotic in the growth medium. L-α,γ-Diaminobutyrate activation was also noted in extracts of *B. polymyxa* ATCC 10401 and *B. circulans* 14040, producers of polymyxin D and circulin, respectively. By contrast, enzyme activity was absent from extracts prepared from several polymyxin-negative and asporogenic mutants derived from *B. polymyxa*. Although these data certainly suggest that the activating enzyme plays a role in polymyxin, circulin, and possibly colistin formation, its direct participation has not been established. No system for the cell-free synthesis of polymyxin has been described, nor is there any evidence that the diaminobutyrate-activating activity is present in extracts of the colistin producer, *B. colistinus*.

SPECIFICITY OF INCORPORATION

In vivo (102, 112, 130, 168) as well as in vitro (30, 33, 34, 37, 58, 63, 93, 97, 120, 128, 153) experiments have revealed that structurally similar amino acids and amino acid analogues can replace certain of the amino acid residues of the peptide chain during antibiotic synthesis. These studies have demonstrated that antibiotic formation is catalyzed by enzymes with rather broad specificities. Multiple forms of an antibiotic are generally produced simultaneously by an organism. These often differ only in the aromatic amino acid or branched-chain amino acid residue that is present at a given site in the peptide. Supplementation with one of these amino acids in vivo or in vitro can direct the biosynthetic process so that the antibiotic(s) formed preferentially will be that rich in the amino acid added exogenously. For example, tyrocidines A, B, and C (differing solely in the number of tyrosine, tryptophan and phenylalanine residues present) are produced in vivo in the ratio of 1:3:7 (102). When tryptophan is added to the medium, *B. brevis* synthesizes principally tyrocidine C and a new tyrocidine, designated D, both of which are rich in tryptophan; neither tyrocidine A or B (phenylalanine-rich) is produced in significant amounts under these conditions. If phenylalanine is supplied instead of tryptophan, there is synthesis of tyrocidines A and B without formation of the C and D components. Provision of both aromatic amino acids leads to the synthesis of tyrocidines A, B, C, and D simultaneously. Amino acid substitutions have also been noted in experiments with the purified gramicidin S and tyrocidine synthetases (33, 34, 37, 63, 93, 97, 120, 128, 153). Thus, tyrosine can replace phenylalanine (position 1) in both antibiotics, whereas tryptophan can substitute for phenylalanine or tyrosine only during formation of the tyrocidine peptides in vitro (34, 128). Additional examples are listed in Tables 3 and 4. In some instances there may be a replacement only at the activation step (i.e., in the formation of the

TABLE 3. *Amino acid and analogue substitutions observed with growing cells*

Antibiotic	Amino acid or analogue added to medium	Comment	Reference
Gramicidin S	DL-Thienylalanine	Replaces D-phenylalanine	168
	DL-Norleucine	Replaces L-leucine to a small extent	168
	DL-F-phenylalanine	Replaces D-phenylalanine to a small extent	168
Tyrocidine	No addition	Tyrocidines A, B, and C formed in ratio of 1:3:7	102, 130
	L-Phenylalanine (D-phenylalanine)	Tyrocidine A synthesized almost exclusively at expense of B and C; tryptophan replaced	102, 130
	L-Tryptophan (D-tryptophan)	Tyrocidine C and D produced; little or no A or B formed; phenylalanine and tyrosine replaced	102, 130
	L-Phenylalanine plus L-tryptophan	Tyrocidines A, B, C, and D synthesized	102, 130
	DL-Thienylalanine	Substitutes for D-phenylalanine	112
	L-Alloisoleucine, L-isoleucine	Substitutes for L-leucine and L-valine	112, 130
	L-Pipecolic acid	Replaces proline to a slight extent	112

TABLE 4. *Amino acid and analogue substitutions observed with cell-free systems*

Antibiotic	Amino acid replaced	Analogue	ATP-PP_i exchange[a] (%)	Antibiotic synthesis[a] (%)	Reference
Gramicidin S	L-Phenylalanine		100	100	74, 97
		D-Phenylalanine	100	105	
		L-Tyrosine	35	1	
		L-Tryptophan	43	0	
		p-F-L-phenylalanine	95	94	
		DL-Threo-β-phenylserine	69	0, 5	
		DL-β-Thienylalanine	89	25	
	L-Phenylalanine			100	128
		L-Tryptophan		0	
		L-Tyrosine		108	
	L-Phenylalanine		100		37
		D-Phenylalanine	71		
		L-Tyrosine	103		
Gramicidin S	L-Proline[b, c]		100	100	93, 97
		Hydroxy-L-proline	0	0	153
		L-Azetidine-2-carboxylic acid	64	Replaces proline	
		Thiazolidine-4-carboxylic acid	<1		
		L-Pipecolic acid	13		
		Trans-3-methylproline	27		
		Trans-4-fluoroproline	1		
Gramicidin S	L-Valine		100	100	93, 97
		D-Valine	1, <1	0	
		L-Isoleucine	18	48	
		D-Isoleucine	9	0	
		L-Alloisoleucine	17	58	
		L-Norvaline	32	105	
Gramicidin S	L-Ornithine		100	100	93, 97
		L-Lysine	2	0	
		L-Arginine	2	0	
		D-Ornithine	<1		
Gramicidin S	L-Leucine		100	100	74, 93, 97
		D-Leucine	19, <1	0	
		L-Isoleucine	20, 16	39	
		D-Isoleucine	10	0	
		L-Alloisoleucine	19	38	
		L-Norleucine	23, 4	22	
Tyrocidine	L-Phenylalanine			100	128
		L-Tryptophan		35	
		L-Tyrosine		95	
	L-Phenylalanine		100	100	34
		DL-Threo-β-phenylserine	102	1	
		DL-β-2-Thienylalanine	82	35	
		DL-*p*-F-Phenylalanine	50	19	
		L-Tryptophan	299	87	
		DL-5-Methyltryptophan	77	52	
		DL-*p*-F-tryptophan	23	33	
	Aromatic amino acids[d]	Standard incubation mixture with L-tryptophan (100 μmol)		Tyrocidine D is major component; small amount of tyrocidine C	34
		Standard incubation mixture with L-tryptophan (1/7 above)		Tyrocidines A, B, and C; smaller amount of D produced	
		Standard incubation mixture minus L-tryptophan		Tyrocidine A formed	
		Standard incubation mixture minus L-tyrosine		Tyrocidine D formed	
		Standard incubation mixture minus L-tryptophan and L-tyrosine		Tyrocidine E synthesized (4-phenylalanine residues; no tyrosine or tryptophan)	

TABLE 4—*Continued*

Antibiotic	Amino acid replaced	Analogue	ATP-PP_i exchange[a] (%)	Antibiotic synthesis[a] (%)	Reference
Tyrocidine	L-Leucine	Isoleucine		Isoleucyl-tyrocidines	34
	L-Leucine		100	100	
		DL-Norleucine	73	11	
Tyrocidine	L-Ornithine	Lysine		Lysyltyrocidines	34
	L-Ornithine		100	100	
		L-Diaminopimelic acid	215	0	
		L-Diaminobutyric acid	5	4	
Tyrocidine	L-Proline		100	100	34
		Hydroxy-L-proline	7	8	
Tyrocidine	L-Valine		100	100	34
		DL-Norvaline	90	5	

[a] 100% is the enzyme activity or antibiotic synthesis obtained with the natural amino acid.

[b] Trans-3-methylproline inhibits L-proline-dependent ATP-PP_i exchange by 29%, whereas under similar conditions trans-4-fluoroproline inhibits the reaction by 93% (93).

[c] The ATP-PP_i exchange reaction with the L-amino acid was inhibited by the corresponding D-enantiomorph as follows: D-valine (80%), D-ornithine (82%), D-leucine (86%), and D-proline (83%) (93).

[d] But see Rao and Hall (120) for strain differences (*B. brevis* ATCC 8185 versus 10068) with respect to effect of aromatic amino acids on cell-free synthesis of tyrocidines A, B, C, and D.

aminoacyl adenylate), suggesting that a greater degree of specificity exists at the point of interaction between the aminoacyl adenylate and the thiol group on the enzyme (97).

RACEMIZATION AND THE SYNTHESIS OF D-AMINO ACIDS

D-Amino acids have been found as constituents of microbial cell walls (142), peptidolipids (3), capsules (161), toxins (46), and antibiotics (14, 20). Racemases for various amino acids have been observed in microorganisms (110); however, the biosynthetic mechanism for the D-amino acid residues in most of these peptide metabolites remains unclear. In some cases, both the L- and D-enantiomorph of an amino acid can be utilized for synthesis of the peptide-bound D-amino acid (71, 160). In other instances, there is evidence to suggest that only the L-isomer is used for the biogenesis of the amino acid in the peptide structure (11, 26, 151). Troy recently reported that a particulate fraction from an encapsulated strain of *B. licheniformis* catalyzed the polymerization of L-glutamic acid (but not the D-isomer) to form a high-molecular-weight poly(α)-D-glutamic acid (161). It has also been observed that the D-amino acid may actually inhibit peptide synthesis, such inhibition being reversed by the L-enantiomorph (26).

Several hypotheses have been advanced to explain the biogenetic origin of D-amino acids in microbial peptides. Mauger (108) has postulated that D-amino acids in antibiotics are formed from L-amino acids after incorporation of the latter into stereochemically labile intermediates such as cyclic dipeptides (e.g., diketopiperazines). Bycroft (20) proposed that a combined (presumably enzyme-bound) form of a dehydroamino acid derived from the corresponding L-amino acid might be reduced stereospecifically in vivo to the D-isomer during antibiotic formation. These hypotheses have not yet been substantiated experimentally. In fact, studies by Huang et al. (54) and Mason et al. (105) indicate that a dehydroamino acid does not participate as an intermediate in the inversion of L-valine to its D-enantiomorph during the respective syntheses of penicillin and actinomycin. By contrast, it now seems certain with respect to tyrocidine and gramicidin S that, after the activation step, racemization of phenylalanine takes place on an enzyme-bound, thioester-linked intermediate (38, 81, 97, 128). Purified light enzyme obtained from either the gramicidin S or tyrocidine synthetase and the intermediate enzyme (tyrocidine synthetase) catalyze the racemization reaction with ATP and L- or D-phenylalanine as substrate (38, 128, 160). The phenylalanine residue at position 1 of the gramicidin S or tyrocidine molecule, as well as the phenylalanine at position 4 of the tyrocidine peptide, is incorporated as the D-amino acid. Recently, Lipmann (95) observed that racemization of phenylalanine labeled with tritium at the α-carbon takes place with complete loss of hydrogen. Details of this experiment have not been published, so the mechanism of the reaction remains somewhat obscure. Likewise, the cell-free synthesis of D-amino acids present in other peptide antibiotics produced by bacilli is not well understood. Ra-

cemization of amino acids may proceed via an analogous mechanism (38, 95).

MUTANT STUDIES

Mutants have frequently provided a useful means to investigate biosynthetic mechanisms and to determine the genetic control of metabolite formation. In this regard, mutants have been obtained that are unable to synthesize a number of peptide antibiotics (e.g., gramicidin S [*B. brevis*] [64, 68, 81, 147], bacitracin [*B. licheniformis*] [43, 44], polymyxin [*B. polymyxa*] [66], and mycobacillin [*B. subtilis*] [121]). Certain of these mutants appear to have defects in structural genes, whereas others may be affected in regulatory sites of the genome which control antibiotic synthetase formation.

Kurahashi et al. (68, 81) and Saito and coworkers (64, 147) characterized several classes of mutants of *B. brevis* which are unable to synthesize gramicidin S. A summary of certain of these mutants is presented in Table 5. Mutants of group I were shown to lack the light enzyme, but possessed a normal heavy enzyme. Group II mutants had a normal light enzyme, but did not contain an L-proline-, L-valine-, L-ornithine-, and L-leucine-activating enzyme activity. Group III mutants lacked both the heavy and light enzyme fractions. As might be expected, cell-free extracts of these mutants were unable to synthesize gramicidin S and could not form D-phenylalanyl-L-prolyl diketopiperazine (i.e., could not initiate peptide synthesis on the heavy enzyme). On the basis of these results, Kambe et al. (68) suggested that different types of mutations may occur, i.e., mutations affecting the regulatory mechanism controlling both light and heavy enzyme synthesis as well as those mutations affecting the regulatory or structural genes of the light or heavy enzyme, individually.

Other types of gramicidin S-less mutants have been described (68, 147). A fourth group appears to have a normal phenylalanine-activating enzyme, but an incomplete or defective enzyme I complex. Characterization of mutants in the fourth group has revealed that the heavy enzyme complex is missing one specific activating enzyme (e.g., for L-proline, L-valine, or L-leucine), but is still able to activate the remaining three amino acids. The fifth group of mutants retained all of the amino acid-activating enzymes needed for gramicidin S formation, but still cannot synthesize gramicidin S. The defect in mutants of groups IV and V may be due to (i) the absence of a subunit that catalyzes the activation of a specific amino acid, (ii) a defect only in the secondary amino acid acceptor site, i.e., the thioester-binding site, or (iii) a conformational change [possibly due to (i)] in the heavy or light enzyme that results in reduced complex formation between the two polyenzyme components. It has also been proposed that other mutational events may influence the interaction and association of the subunits of the heavy enzyme or the formation of the phosphopantetheine arm (68).

Haavik and Frøyshov (43) recently isolated a mutant, obtained from *B. licheniformis* AL, that was unable to produce bacitracin as a result of a defective enzyme complex. As described earlier (30, 56), the parental strain produces a bacitracin synthetase that can be separated into three complementary fractions, A, B, and C; all three components are needed for bacitracin synthesis. Only two fractions (AC_0 and B_0) were obtained from the mutant, *B. licheniformis* SB319 (43). Component AC_0 activated the same amino acids as components A and C from the parent. B_0 activated the same amino acids as the parental B fraction. However, there were marked differences seen in the

TABLE 5. *Properties of some gramicidin S-less mutants of Bacillus brevis*

Mutant class	Amino acid-activating enzyme for:					Ability to form:	
	Phenylalanine	L-Proline	L-Valine	L-Ornithine	L-Leucine	Gramicidin S	DKP[a]
None, wild type	+	+	+	+	+	+	+
I	−	+	+	+	+	−	−
II	+	−	−	−	−	−	−
III	−	−	−	−	−	−	−
IV	+	−	+	+	+	−	−
	+	+	−	+	+	−	+
	+	+	+	+	−	−	+
	+	+	+	+	−	−	−
V	+	+	+	+	+	−	+
	+	+	+	+	+	−	−

[a] DKP, D-Phenylalanyl-L-prolyl diketopiperazine.

degree of the ATP-PP_i exchange reaction with the enzyme fractions derived from the producer and nonproducer cells. Mixing experiments revealed that enzyme component B_0 could catalyze the synthesis of bacitracin with components A and C; by contrast, component AC_0, when used with component B, was unable to synthesize the antibiotic. The evidence, therefore, suggests that the bacitracin synthetase obtained from the nonproducer was defective and that the lesion may involve the activation sites for the amino acids. The affinity between the enzyme components may also be affected.

POSSIBLE FUNCTIONS OF PEPTIDE ANTIBIOTICS IN THE PRODUCING ORGANISM

The function of antibiotics in the metabolism of the producing organism has been the subject of considerable speculation and discussion (17, 18, 164, 166, 170). However, there has been little understanding with respect to the role, if any, that they actually do play. Several proposed functions for antibiotics are no longer accepted as likely candidates, whereas others are still under consideration in several laboratories. Those that have been discarded involve antibiotics as evolutionary relics, waste products of cellular metabolism, reserve food materials, spore coat components, or breakdown products derived from cellular macromolecules (166). Still under current consideration (40) is the possibility that antibiotics function to kill or inhibit the growth of other organisms in nature, thereby providing a competitive advantage to the producing species (17). Although one usually thinks of this competition as between bacterial species A versus bacterial species B or bacterium versus fungus, it could involve bacteria versus amoebae (S. H. Hutner, personal communication) since these protozoa use bacteria as sources of food (150). Another possibility is that the competition exists between strains of the antibiotic-producing species itself (101; E. Rosenberg, personal communication). A further variation of the competition hypothesis involves the excretion of the antibiotic during spore germination in order to eliminate competitors in the immediate environment of the germinating spore. A completely different concept, proposed by Bu'Lock (18), is that antibiotic synthesis is a means of keeping the cellular machinery in working order during the time when cell growth is not possible due to unfavorable conditions. Although this is an interesting speculation, no data exist in support of the maintenance hypothesis. Additional hypotheses currently being debated state that synthesis of an antibiotic (or other secondary metabolites) is a method of avoiding cell death due to unbalanced growth (166), or that it provides a mechanism of detoxification (170). With respect to the unbalanced growth hypothesis, it is assumed that certain primary metabolites (e.g., amino acids or nucleotides) are overproduced, when conditions for exponential growth are no longer favorable, due to ineffective control systems in the producing organism. These metabolites are thought to be converted to antibiotics which are released from the cell. The detoxification hypothesis is slightly different, proposing that certain toxic antimetabolites are synthesized when exponential growth terminates, e.g., analogues of nucleosides, amino acids, or amino sugars. Conversion to the antibiotic is thought to provide a method of detoxification. Inherent in both the unbalanced growth and detoxification proposals is the assumption that the antibiotic is not toxic to the producing organism (170). There exists no evidence in support of these two hypotheses. Indeed, most antibiotics are more toxic to the producing organism than are their precursors (24). Other possibilities for antibiotic function include (i) facilitation of metal transport into cells (43) and (ii) autoinhibition of germination (29). The latter is suggested by the following observations: many microorganisms produce self-inhibitors of germination (154); various antibiotic producers contain the antibiotic in their spores (27, 29, 67).

Much of the criticism against the competition hypothesis is based on the inability of certain investigators to detect antibiotic production in unsterilized soil which has not been supplemented with organic nutrients (164). The same type of criticism has also been used to support the viewpoint that antibiotics are biological accidents which are of no direct benefit to the cell. However, it is known that soil which contains seeds and decaying vegetable matter and the rhizosphere support antibiotic producion (17, 19, 40, 78).

Of the various functions postulated for antibiotics, the one which has received the most attention in recent years is the view that antibiotics are important compounds in cellular differentiation, i.e., the transition from vegetative cells to spores (52, 55, 132, 139). Numerous antibiotics are elaborated by microorganisms that undergo sporulation. Due to the fact that the vast majority produced by bacilli are peptidic in nature, these peptides have been singled out as playing a key role in the termination of vegetative growth, allowing sporulation to take place. The following factors make this hypothesis attractive (24, 45). (i) Practically all sporulating

microorganisms produce antibiotics. (ii) Although it was once believed that the antibiotics elaborated by an organism have no effect on the producing cell, it is now well established that they are inhibitory to vegetative growth at concentrations at which they are generally produced by sporulating cultures (65, 123, 138, 152). Peptide antibiotics produced by bacilli specifically inhibit important cellular processes, e.g., deoxyribonucleic acid (DNA) synthesis, cell wall synthesis, membrane function, and structure (52, 132). (iii) Production of peptide antibiotics usually begins at the late-logarithmic phase of growth and continues during the early stages of the sporulation process in bacilli (5, 10, 11, 36, 82, 115, 138, 160). (iv) Sporulation and antibiotic synthesis are induced by depletion of some essential nutrient. (v) There are genetic links between the synthesis of antibiotics and the formation of spores (24, 45, 139). For example, antibiotic formation by certain bacilli is an early event beginning at stage I of sporulation. Mutants that are asporogenous (and map at different loci) are almost always antibiotic negative and, when so, are blocked very early (66, 68, 143). Revertants, transductants, and transformants of stage 0 asporogenous mutants, restored in their ability to synthesize antibiotic, also regain the ability to sporulate (4, 51, 140). Also, conditional asporogenous mutants fail to produce antibiotic at the nonpermissive temperature (92, 158). (vi) Physiological correlations also favor a relationship between the production of an antibiotic and spore formation. As an example, inhibitors of sporulation also inhibit antibiotic synthesis (10, 65, 115). Furthermore, both processes are repressed by glucose (10, 139), and concentrations of manganese ion of at least two orders of magnitude higher than that required for normal cellular growth are needed for sporulation and antibiotic synthesis by certain species of *Bacillus* (165).

Because of the above observations, several investigators have concluded that antibiotic production is required for sporulation. Hodgson (52) speculated that peptide antibiotics might be used in several ways by an organism during the process of sporulation as modifiers of the cell membrane, e.g., as detergents disrupting structural components, or as ion carriers, modifying permeability properties. Hodgson argued that, by selectively functioning at certain stages, the sporulation process would be able to proceed normally. Sadoff (132) pointed out that the peptide antibiotics produced by bacilli exhibit effects upon membrane synthesis and function, cell wall synthesis, and nucleic acid synthesis, which are important processes for sporulation. He contended that the antibiotics, when produced by an organism, may act as selective modifiers of cell function—i.e., repressing or inhibiting vegetative cell macromolecular synthesis, but permitting endospores to be formed.

Although the above observations and conclusions point to an intimate relationship between antibiotic formation and sporulation, by no means do they prove that the antibiotic is necessary for sporulation. Furthermore, there are data which fail to support this hypothesis. For example, there are conditions in which increased sporulation is accompanied by a decrease in antibiotic production (143) and vice versa (11). Recent nutritional studies on gramicidin S formation by *B. brevis* revealed a dissociation between sporulation and antibiotic formation (25). Media used at the beginning of the investigation supported sporulation but yielded little to no gramicidin S. As more suitable media were devised for antibiotic production, sporulation became markedly poorer. Another indication of a physiological dissociation between gramicidin S formation and sporulation arose from chemostat experiments in which nutrient limitation was used to study formation of the light and heavy gramicidin S synthetases (106). The enzymes were produced at intermediate growth rates under carbon, nitrogen, sulfur, or phosphorus limitation. Although carbon limitation yielded the poorest synthetase activity, it was the only condition under which significant amounts of spores were formed. Also, the growth rate favoring sporulation was considerably lower than that which was optimal for gramicidin S synthetase formation.

The most damaging evidence to the hypothesis involving antibiotics in sporulation is the existence of mutants which are antibiotic negative but can still sporulate. For example, Kambe et al. isolated gramicidin S-negative mutants of *B. brevis* with genetic blocks in the formation of the heavy synthetase, the light synthetase, or both (68). Upon examining the sporulating abilities of the gramicidin S-negative mutants, Kambe et al. (68) found them all to be oligosporogenous. However, recent studies on mutant hh, reportedly containing a specific defect in the leucine-activating function of the heavy synthetase (68), revealed that this gramicidin S-negative mutant sporulates normally (25).

The ability of peptide antibiotic-negative mutants to sporulate is also seen with other bacilli. Adenine-requiring mutants of *B. subtilis* B_3, which fail to produce intra- or extracellular mycobacillin, sporulate and germinate very well, the spores containing normal concentrations of dipicolinate (121). Similarly, *B. licheni-*

formis mutant SB 319 produces no detectable intra- or extracellular bacitracin, yet it sporulates normally (44). Both parent and mutant have the same growth rate, morphology, and biochemical characteristics, but the mutant has a defective bacitracin synthetase complex (43).

Much of the enthusiasm in favor of a function of antibiotics in sporulation derives from the work of Sarkar and Paulus (138). They noted a specific inhibition of RNA synthesis by the tyrothricin complex during growth of the producing culture, *B. brevis* ATCC 8185. Moreover, purified *B. brevis* RNA polymerase was also inhibited by the complex. Both components of the tyrothricin complex (linear gramicidin and tyrocidine) but not gramicidin S, produced by another strain of *B. brevis*, were shown to exhibit similar inhibitory activity, indicating the specificity of the effect. They also observed that the cessation of exponential vegetative growth (t_0) was accompanied by a sharp decline of net RNA synthesis. Tyrothricin synthesis began at t_0; the antibiotic complex remained largely associated with the cell material and reached a maximum level 2 h after exponential growth (t_2). Sarkar and Paulus (138) advanced the view that peptide antibiotics regulate gene transcription during the transition from vegetative growth to sporulation by selectively terminating the expression of vegetative genes. Unfortunately, they erroneously claimed that all peptide antibiotics were produced by sporulating microorganisms, a statement that ignores the production of comirin by *Pseudomonas antimycetica*, micrococcin P by *Micrococcus* sp., and nisin by *Streptococcus lactis* and *Streptococcus cremoris*. It is even more disconcerting to read (65, 141) that *all* antibiotics are made by sporulating cultures!

Kleinkauf and co-workers (122, 123, 141) also proposed a role for tyrocidine and linear gramicidin in *B. brevis* sporulation. Their data suggest opposing effects of the two peptides in vivo. In vitro, tyrocidine inhibited transcription by forming a complex with DNA. Although linear gramidicin also inhibited transcription, it did not complex with DNA. Of particular interest was the ability of linear gramicidin to abolish the action of tyrocidine, allowing RNA synthesis to proceed. Tyrocidine also inhibited in vivo transcription in *B. brevis* cells; in the presence of linear gramicidin, however, a higher concentration of tyrocidine was required to attain complete inhibition. To explain these data, Ristow et al. (122) proposed that in vivo a complex may be formed between linear gramicidin and tyrocidine which would have less affinity for DNA than tyrocidine alone. They argued that perhaps both peptides are required to ensure that not all of the genes are turned off at the end of vegetative growth so that some can be transcribed during sporulation. What is disturbing about this hypothesis are their data showing that, although linear gramicidin reversed the effect of tyrocidine on growth and in vivo RNA synthesis, it did not reverse tyrocidine's inhibition of sporulation; in fact, in the presence of the antibiotic mixture (i.e., in the proportion in which they are made as the tyrothricin complex), sporulation was completely inhibited. To us, this means that the inhibition of RNA synthesis has nothing to do with sporulation. The main conclusions that one can derive from these studies is that both tyrocidine and linear gramicidin inhibit *B. brevis* RNA polymerase. However, this is not specific nor surprising since this group (141) has shown that tyrocidine also inhibits *E. coli* RNA polymerase, and the effect on *B. brevis* polymerase is also exerted when calf thymus DNA is substituted for *B. brevis* DNA as template.

All of the experimental data described to date could fit in well with the hypothesis that sporulation and antibiotic formation, although independent phenomena, are regulated by a common (or a similar) regulatory mechanism. Many of the mutants used in obtaining the data suggesting a role of antibiotics in sporulation were pleiotropic and probably blocked in regulatory genes. An attempt to demonstrate a function for the antibiotic in sporogenesis by adding crude antibiotic to pleiotropically asporogenous and antibiotic-negative *Bacillus* mutants failed to induce sporulation (139). As suggested by Schmitt and Freese (143), the critical experiment involving the isolation of an asporogenous mutant that can sporulate only upon addition of pure antibiotic has never been reported. The closest to such a finding was the claim of Jayaraman and Kannan (65) that addition of tyrothricin to the sporogenic *B. brevis* ATCC 8185 culture inhibited growth but enhanced sporulation. However, Ristow et al. (123) failed to confirm this result. Jayaraman and Kannan (65) also reported that addition of polymyxin to its producer, *B. polymyxa*, inhibited growth and stimulated sporulation. However, the significance of this observation is obscured by their finding that the antibiotic also inhibited glucose uptake. Thus, sporulation could have been enhanced merely by release of sporulation from catabolite repression. When D-leucine, an inhibitor of polymyxin synthesis (but not growth), was supplied to *B. polymyxa*, sporulation was strikingly inhibited. L-Leucine reversed the effect of D-leucine on both antibiotic production and sporulation. However, since L-leucine is needed for both polymyxin synthesis and for protein synthesis during sporulation, the effect of D-leucine

can be explained by an inhibitory effect on two independent phenomena.

An oft-quoted and misquoted (65, 122, 123, 141) finding in support of a sporulation function for antibiotics are the data of Yoshida et al. (173), which revealed that addition of actinomycin to young cultures of the producer organism (*Streptomyces antibioticus*) markedly inhibited protein synthesis, whereas later addition during actinomycin production had no effect. It is claimed by these investigators (65, 122, 123, 141) that, since the antibiotic exerts its effect during growth but not during the stationary phase, it plays an important role in the transition between a growing and a differentiating cell. However, the subsequent finding (104) that *S. antibioticus* becomes impermeable to exogenous actinomycin when the organism enters the stationary phase has apparently been missed by those who quote the original observation and surely invalidates their claim.

Some recent experiments by Lee et al. (90) have provided an additional, although as-yet-unexplained, facet to the present controversy. It has been known that the enzymes for tyrocidine synthesis suddenly appear at the end of the exponential growth phase; only during a short period at the onset of the stationary phase has it been possible to obtain active soluble enzyme extracts. Later in the stationary phase, only particulate enzymes are recovered. Biochemical investigations carried out concomitantly with electron microscopy have shown most of the tyrocidine-forming activity becomes associated with forespores in *B. brevis*. The significance of the migration of antibiotic-synthesizing activity from the soluble portion of the cell into the forespores is not clear. Perhaps it merely represents a nonspecific trapping of vegetative cell components that are truly unessential to the sporulation process or to the spore itself. The authors (90) are properly cautious in their interpretation of these results, stating that their data are "consistent with the assumption of an effect of the antibiotic facilitating the transition from the vegetative phase into sporulation. However, this laboratory has not yet probed into the causative connection between antibiotic and spore formation."

It should be clear to the reader that the mystery of antibiotic function has not been solved. We feel, however, that peptide antibiotics must have some role in the survival of the producing organism. It is inconceivable to us that the intricate reaction sequences of antibiotic biosynthesis would have been retained in nature without benefit to the organism. It is often argued that the strain degeneration problem in the antibiotic industry speaks against a survival function. However, this argument is irrelevant since the amounts produced in commercial antibiotic fermentations are many orders of magnitude greater than that needed for an organism's survival. In fact, the abnormal degree of synthesis observed in industrial fermentations is probably detrimental to the well-being of the organism and it is logical to assume that the culture would revert to a lower-producing, more rapidly growing form which would be capable of more balanced metabolic activity.

Whatever the true function of antibiotics, many mechanisms exist whereby organisms can protect themselves from the antibiotics they elaborate (24). Permeability changes, compartmentalization, and the presence of an inactive form of the antibiotic intracellularly may all play a role in preventing self-annihilation. In support of the idea of "a built-in survival kit," Kurylo-Borowska (83) demonstrated in *B. brevis* Vm4 that edeine B synthesized in vivo is present in an inactive form covalently bound to the edeine-synthesizing polyenzymes and associated with a complex of cytoplasmic membrane and DNA. Free edeine is not found in the cells and the polyenzyme-membrane-DNA-bound edeine neither exhibits antimicrobial activity nor influences DNA synthesis. Biological activity is seen only when the antibiotic is released from the cell or from the complex in vitro.

CONCLUDING REMARKS

It is now evident that the biosynthesis of peptide antibiotics by members of the genus *Bacillus* is directed by multienzyme thiotemplates. Amino acids employed as substrates for antibiotic synthesis are activated by ATP to form enzyme-bound aminoacyl adenylates. The aminoacyl moiety is then transferred to secondary acceptors, i.e., to specific SH groups, to form covalent thioester bonds. Antibiotic synthesis involves polyenzyme complexes that exhibit a broader specificity than that shown by ribosomal protein-synthesizing systems. tRNA, mRNA, and ribosomes are not directly involved. In antibiotic synthesis, the polyenzyme complex serves as template for the proper sequencing of the amino acids in the peptide. The processes of initiation, elongation, and termination are inherent properties of the polyenzyme systems. The energy derived from the thioester bonds provides the driving force for the peptidation reactions. As observed in the growth of ribosomal polypeptide chains, antibiotic formation is initiated from the N-terminal position and ends at the carboxyl terminus. In the case of antibiotics such as gramicidin S and tyrocidine, cyclization is the final step before release of the antibiotic from the enzyme. This reaction, as well as the mechanism of amino acid racemization, is not well under-

stood. 4'-Phosphopantetheine plays an important role as coenzyme in the process of peptide chain elongation, mediating alternative transthiolation and transpeptidation reactions.

Little progress has been made in elucidating the function(s) of antibiotics in the producing organism. We believe that they indeed have a function(s) and that they are made in nature, especially in microenvironments containing organic matter. It is doubtful that all antibiotics have the same general function; we certainly accept the fact that different classes of proteins have different functions.

The view that peptide antibiotics function in cellular differentiation (i.e., sporulation) is not supported by data obtained with producers of gramicidin S, mycobacillin, and bacitracin. In these cases, nonproducing mutants are capable of sporulation. Of course, not enough work has been done to eliminate the possibility that other peptide antibiotics are involved in sporulation.

If the antibiotic is not an obligatory part of the sporulation process, then why are antibiotic production and sporulation so often related to one another? We propose that they are regulated by the same or similar control mechanisms and, furthermore, that there are reasons why it is beneficial for the sporulating cell to produce an antibiotic. For example, (i) the antibiotic could be packaged in the *Bacillus* spore and excreted during germination to inhibit or kill competitors, thereby providing an environment favorable for the development of the germinating spore; or (ii) the antibiotic could be packaged in the spore and inhibit germination until environmental conditions become more favorable. It is hoped that the availability of nonproducing *Bacillus* mutants will now allow us to approach the problem of antibiotic function in a more conclusive manner.

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ADDENDUM IN PROOF

Akers et al. (1b) recently reported that two enzyme fractions (derived from *B. brevis*) catalyzed the synthesis of gramicidin A to the heptapeptide stage. By contrast, Akashi et al. (1a) noted that the synthesis of formylvaline and formylvalylglycine could be achieved employing a partially purified enzyme system from *B. brevis* ATCC 8185. The enzyme preparation (component I) catalyzed an ATP-PP_i exchange reaction that was dependent solely on valine and glycine. The molar ratio of valine to glycine bound by component I was approximately 1. Evidence was also obtained that the amino acids were bound to component I by thioester linkage and that formylvaline and formylvalylglycine were synthesized (in the presence of a formyltetrahydrofolate-synthesizing system, ATP, valine, glycine, and an "AS45" fraction from *B. brevis*) as enzyme-bound intermediates. The authors (1a) proposed the following biosynthetic sequence for gramicidin A formation: (i) initiation via the formylation of valine bound to component I, followed by the synthesis of formylvalylglycine; (ii) peptide elongation through transfer of formylvalylglycine to a second enzyme component that activates and binds alanine and leucine; (iii) further elongation of the nascent peptide, which may require additional enzyme components. The biochemical mechanism for linking ethanolamine to the C-terminal tryptophan residue of gramicidin A still remains undetermined.

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